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TITLE: Secreted Wnt Signaling Inhibitors in Disuse-Induced Bone Loss

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> <p>To investigate whether antagonizing secreted inhibitors of Wnt signaling is a viable approach for combating the bone loss that normally accompanies neuromuscular paralysis, we have conducted several paralysis experiments in mice that address the potential role of sclerostin inhibition in preventing paralysis-induced bone loss. In the first series, adult female mice were subjected to unilateral Botox-induced muscle paralysis of the lower limb via Botox (20 U/kg) injection into the right lower limb musculature. We performed this treatment in 2 types of mice: Wild type (Sost<sup>+/+</sup>) and Sost knock-out mice (Sost<sup>-/-</sup>). Despite the equal loss in muscle mass induced by Botox, Sost<sup>+/+</sup> mice lost a significant percentage of their initial lower-limb aBMD and BMC over the experimental period, whereas bone mass in Sost<sup>-/-</sup> mice actually increased significantly (~2-4%; p&lt;0.05) over the same period. Similar effects were seen for BV/TV, trabecular thickness, and trabecular bone mineral content (Tb.BMC) in the distal femur. We also investigated the osteopenic effects of Botox-induced muscle paralysis in WT mice that were given neutralizing antibody to sclerostin. Botox injection into the right limb musculature of vehicle-treated mice resulted in an 8.4% decrease (p&lt;0.001) in femoral aBMD, whereas mice given Scl-AbIII after Botox injection exhibited an 8.0% increase in femoral aBMD in the paralyzed limb. In summary, these data suggest that sclerostin inhibition is a useful approach for overcoming the bone loss that normally occurs with disuse.</p>					
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## INTRODUCTION

Osteoporosis (porous bone disease) is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures. Disuse osteoporosis occurs when the normal loading environment experienced by bone cells is reduced or removed. A decreased mechanical loading environment (e.g., as occurs in soldiers after spinal cord injury) leads to rapid bone loss *via* enhanced local osteoclastic bone resorption, concomitant with a suppression of bone formation. In the funded application, we proposed to determine whether local, secreted regulators of Wnt/Lrp signaling (Sost, Dkk1) modulate bone loss in response to mechanical disuse. Furthermore, we proposed to test whether these molecules can be manipulated to prevent bone loss that normally accompanies disuse. To accomplish this goal, we proposed to induce disuse (using Botox-induced paralysis of the quadriceps, hamstrings, and soleus) in one hindlimb of a series of mice with mutations in Wnt modulators (Sost<sup>-/-</sup>, Dkk1<sup>+/-</sup>) and in wild-type mice that are also treated with neutralizing antibody to Dkk1 or Sost (or both). These experiments have the potential to reveal new treatment strategies for overcoming the disuse-associated bone loss that accompanies spinal cord injury, and other battlefield-related injuries resulting in neuromuscular impairment.

## BODY

In the first year of the project, we have completed several tasks outlined in the original application. Task 1 was to gain IACUC approval for the experiments proposed. We accomplished this task, gaining approval at both the Indiana University School of Medicine and at the Department of Defense, which required a separate IACUC (ACURO) application.

We have completed most of the Sost-related tasks in this project. This includes Subtask 2.1, 3.1, 3.2 3.3, 6.1, and task 7. Those results are described below.

### ***Sclerostin knockout mice are protected from the bone-wasting effects of muscle paralysis.***

Adult male Sost<sup>+/+</sup> and Sost<sup>-/-</sup> mice were raised to 25 wks of age, at which point they were subjected to unilateral Botox-induced muscle paralysis of the lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscle paralysis for three

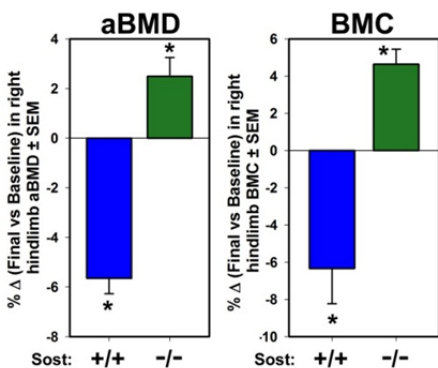


Figure 2: Sost<sup>+/+</sup> lost a significant portion of their BMD and BMC as a result of Botox treatment, but Sost<sup>-/-</sup> mice actually gained bone during muscle paralysis.

weeks. Impaired limb function was apparent 2 days after Botox

treatment, as revealed by a failure of the foot to cling to a support when the mouse was inverted. Prior to Botox treatment, the mice were given whole-body scans via high-resolution DEXA using a pixiMUS mouse densitometer. Mice lost a slight significant (~13-15%, P<0.05) percentage of their body mass over the experimental period (Fig 1, left), but Sost genotype had no effect on their weight loss. Likewise, Botox treatment caused significant muscle atrophy in both genotypes (~47-49% loss; p<0.001), as assessed by quadriceps weight at sacrifice from Botox-treated vs. saline treated limbs (Fig. 1, right). Despite the equal loss in muscle mass, Sost<sup>+/+</sup> mice lost a

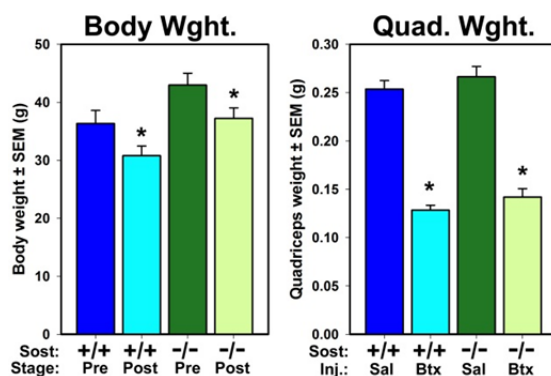


Figure 1: Sost<sup>+/+</sup> and Sost<sup>-/-</sup> treated with Botox in the right lower limb lost equal amounts of body mass over the experimental period. Botox treatment also induced equal amounts of muscle wasting in the two genotypes.

significant percentage of their initial lower-limb aBMD and BMC over the experimental period, whereas bone mass in  $Sost^{-/-}$  mice actually increased significantly ( $\sim 2-4\%$ ;  $p < 0.05$ ) over the same period (Fig 2). This trend in the  $Sost^{-/-}$  mice was also observed in the saline treated limb, suggesting that the normal increase in bone mass in adult male  $Sost^{-/-}$  mice was not hampered by disuse. We next examined bone loss in the distal femoral metaphysis by comparing saline-treated to Botox-treated femora after sacrifice (Fig. 3). Bone volume fraction (BV/TV), trabecular thickness, and trabecular bone mineral content (Tb.BMC) were significantly decreased ( $\sim 18-28\%$ ;  $p < 0.01$ ) by Botox treatment in  $Sost^{+/+}$  mice, but those same trabecular properties were unchanged by Botox treatment in  $Sost^{-/-}$  mice. Next, we extracted mRNA from whole tibias that were snap-frozen in liquid  $N_2$  immediately after sacrifice. We performed real-time PCR reactions for genes associated with osteoclastic resorption regulation (Fig. 4). Disuse resulted in no change in M-csf in either genotype. Osteoprotegerin was reduced significantly by Botox treatment in both genotypes, whereas RankL expression was significantly enhanced in the paralyzed limbs of  $Sost^{+/+}$  but not  $Sost^{-/-}$  mice.

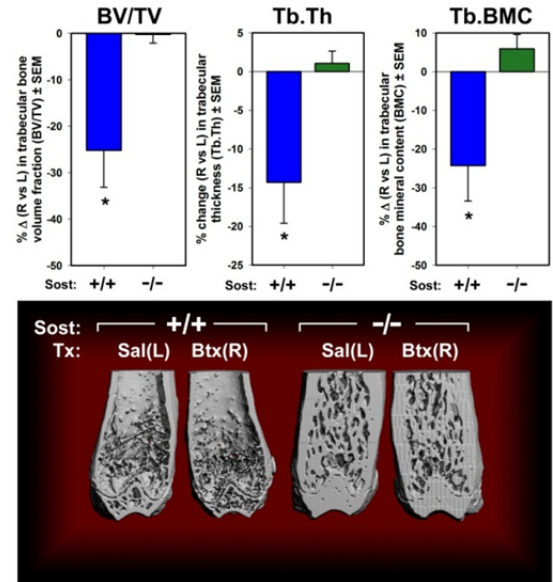


Figure 3: Bone volume fraction in the distal femoral metaphysis was reduced by Botox treatment in  $Sost^{+/+}$  mice, but not in  $Sost^{-/-}$  mice.

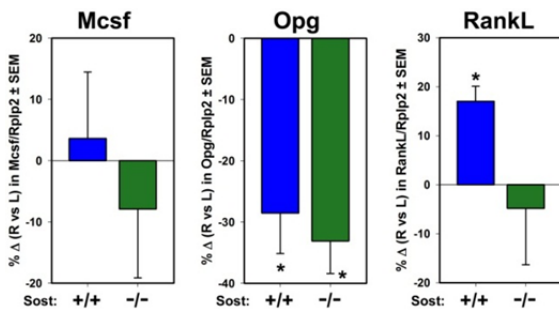


Figure 4: Real-time PCR evaluation of transcripts related to the resorption axis revealed no Botox-induced changes in M-Csf expression, significant downregulation of the decoy receptor Opg in both  $Sost^{+/+}$  and  $Sost^{-/-}$  mice, and significant upregulation of RankL expression in  $Sost^{+/+}$  but not  $Sost^{-/-}$  mice.

### ***Sclerostin-neutralizing antibody protects the normal (wild-type) skeleton from disuse-induced bone loss.***

Adult female Swiss-Webster mice were subjected to unilateral botox-induced muscle paralysis of the lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscular paralysis for three weeks in the presence or absence of twice-weekly dosing with a sclerostin neutralizing antibody (Scl-AbIII; 25 mg/kg) injection. Prior to Botox treatment, the mice were scanned through the proximal and midshaft tibia using high-resolution pQCT (Stratec SA+), and a whole-body DEXA scan was collected using high resolution DEXA (pixiMUS) prior to disuse onset, and again at sacrifice. Mice receiving Scl-AbIII exhibited a slight (5%) but significant increase in body weight over the 3-wk treatment period, whereas mice receiving vehicle control injections

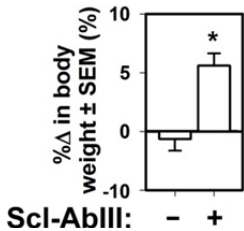


Figure 5: Body weight was not changed by twice-weekly saline injection, but Scl-AbIII injection led to increased body mass.

lower limb via botulinum toxin (Botox; 20 U/kg) injection into the quadriceps, hamstrings, triceps surae, and leg extensor compartment of the right lower limb. The left lower limb was injected identically with saline (internal control). The mice underwent unilateral muscular paralysis for three weeks in the presence or absence of twice-weekly dosing with a sclerostin neutralizing antibody (Scl-AbIII; 25 mg/kg) injection. Prior to Botox treatment, the mice were scanned through the proximal and midshaft tibia using high-resolution pQCT (Stratec SA+), and a whole-body DEXA scan was collected using high resolution DEXA (pixiMUS) prior to disuse onset, and again at sacrifice. Mice receiving Scl-AbIII exhibited a slight (5%) but significant increase in body weight over the 3-wk treatment period, whereas mice receiving vehicle control injections

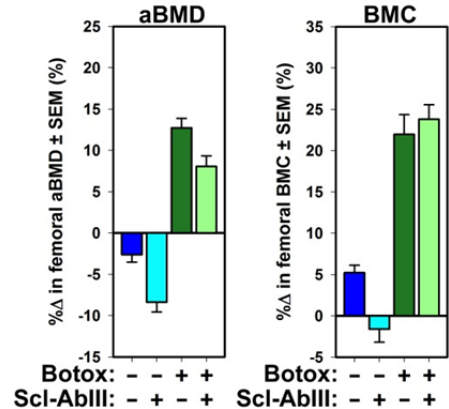


Figure 6: Swiss Webster mice exposed to unilateral Botox treatment were spared the loss of BMD and BMC in the affected limb if they were treated with 25 mg/kg Scl-AbIII twice weekly during the disuse phase.

had no change in weight (Fig. 5). Saline injection into the left lower limb musculature resulted in a slight but significant decrease in femoral aBMD (-2.6%,  $p < 0.01$ ) over the 3-wk treatment period, whereas

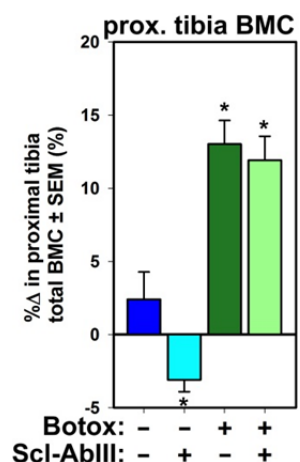


Figure 7: Botox treatment resulted in a significant loss of BMC in the trabecular-rich proximal tibia, but the same disuse conditions actually resulted in increased BMC when Scl-AbIII was given.

Botox injection into the right limb musculature of these same animals resulted in an 8.4% decrease ( $p < 0.001$ ) in femoral aBMD (Fig. 6). Mice given Scl-AbIII during the 3 wk period following Botox injection exhibited an 8.0% increase in femoral aBMD in the paralyzed limb. This increase was significantly different from the changes occurring in the saline-injected limbs of mice not receiving antibody ( $p < 0.001$ ), suggesting that Scl-AbIII not only protected these paralyzed limbs from bone loss, but actually increased bone mass over non-paralyzed, saline-injected limbs. Similar effects were noted for femoral BMC. We were unable to detect any Botox-related changes in the distal femur metaphyseal trabecular bone using  $\mu$ CT scanning, and therefore the Scl-AbIII effects on disuse were not amenable to evaluation using this technique. However, we did take baseline

and post-treatment pQCT slices through the proximal tibia, which is a trabecular-rich site, and found a significant Botox effect (~3.5% loss), which was nullified by Scl-AbIII treatment (Fig. 7). In summary, these data suggest that sclerostin inhibition is a useful approach for overcoming the bone loss that normally occurs with disuse/paralysis.

### ***Effects of Dkk1 inhibition on the bone-wasting effects of muscle paralysis***

We have begun treating mice with neutralizing antibody to Dkk1 (Dkk1-mAb). Our study design is similar to that explained in the previous section for the Scl-AbIII studies. We are in the middle of our treatment period as I write this report. We hope to have all of the data analyzed by the late fall 2011.

### **KEY RESEARCH ACCOMPLISHMENTS**

- We determined that genetic deletion of the Sost gene protected mice from the bone-wasting effects of muscle paralysis.
- We determined that Sost deletion prevents the paralysis-induced upregulation of RankL expression, which normally accompanies disuse.
- We determined that adult-onset sclerostin inhibition (via neutralizing antibody) prevented the bone-wasting effects of muscle paralysis in normal (wild-type) mice.
- We determined that anti-sclerostin therapy might actually induce *increased* bone formation in the presence of mechanical disuse.
- Our preclinical studies indicate that sclerostin inhibition might be a useful therapy for soldiers that have experienced muscle paralysis, in order to maintain bone mass in the rehabilitating limb.

### **REPORTABLE OUTCOMES**

Data from the experiments described above have been presented at last year's (Nov., 2010) American Society for Bone and Mineral Research meeting, held in Toronto, Canada.

I will be presenting these data at two other conferences coming up this summer (June 2011). One is the “Genetics and Evolution of the Skeleton Research Initiative” (GESRI) held at UCSF. The second is a Gordon Conference on Bones and Teeth, held in Les Diableretes, Switzerland. In these talks, I have and will continue to attribute my funding source for the data to the DOD’s CDMRP.

None of the data have yet been published. I plan to begin writing up the Sost genetic study and the sclerostin antibody study as one paper, and the target date for submission is July 2011.

## **CONCLUSIONS**

Our experiments, to date, support the hypothesis that sclerostin inhibition can significantly reduce, or perhaps even eliminate, the bone-wasting effects of mechanical disuse that occur following neuro-muscular paralysis.

*“So what?”*

These pre-clinical results are important because many soldiers returning from Afghanistan present with spinal cord injuries (SCI) or other peripheral nerve injuries that induce paralysis. Disuse osteoporosis is a common sequelae of spinal cord injury (SCI) and peripheral nerve damage. Bone mineral content (BMC) can decrease by as much as 70% following SCI. This bone loss results in increased bone fragility and a subsequent increase in the risk for low-trauma fractures. Disuse osteoporosis is a particularly debilitating disease for soldiers in whom recovery of neuromuscular function is possible, because hard-fought gains in neuromuscular rehabilitation can be lost if the underlying bony structure has deteriorated to the point where muscle activity induces fractures. If this situation arises, neuromuscular training must cease to allow the fracture to heal. Our experiments suggest that targeting sclerostin might be a therapeutic approach to accompany neuromuscular rehabilitation, because it appears to preserve the bone mass and structure in paralyzed limbs, ultimately providing a foundation for neuromuscular recovery.

## **REFERENCES**

N/A

## **APPENDICES**

N/A

## **SUPPORTING DATA**

N/A